AMMONIATED GLYCYRRHIZIN

Effects of Ammoniated Glycyrrhizin on Blood Pressure, Electrolytes and Corticosteroids in various Strains of Rats

By

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REPORT ON MASTER'S THESIS

This is to certify that we have examined the Master's thesis submitted by Janette Robuck Taylor entitled Effects of Ammoniated Glycyrrhizin on Blood Pressure, Electrolytes and Corticosterone in Various Strains of Rats and recommend its acceptance in satisfaction of the thesis requirement for the degree of Master of Arts (Science).

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EFFECTS OF AMMONIATED GLYCYRRHIZIN ON BLOOD PRESSURE, ELECTROLYTES AND CORTICOSTEROIDS IN VARIOUS STRAINS OF RATS

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Master of Science

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
AG	Ammoniated glycyrrhizin
DOC	11-deoxycorticosterone
DOCA	Deoxycorticosterone acetate
DPM	Disintegrations per minute
GLC	Gas-liquid chromatography
i. p.	Intraperitoneal
i. v.	Intravenous
KRBG-A	Krebs-Ringer-Bicarbonate with glucose and albumin
O. M.	Osborne-Mendel
pure-AG	Ammonium glycyrrhizinate
s. c.	Subcutaneous
S. D.	Sprague-Dawley
SEM	Standard error of the mean
S. H.	Spontaneous Hypertensive
tech-AG	Ammoniated glycyrrhizin, technical grade
TLC	Thin layer chromatography

CHAPTER I

INTRÓDUCTION

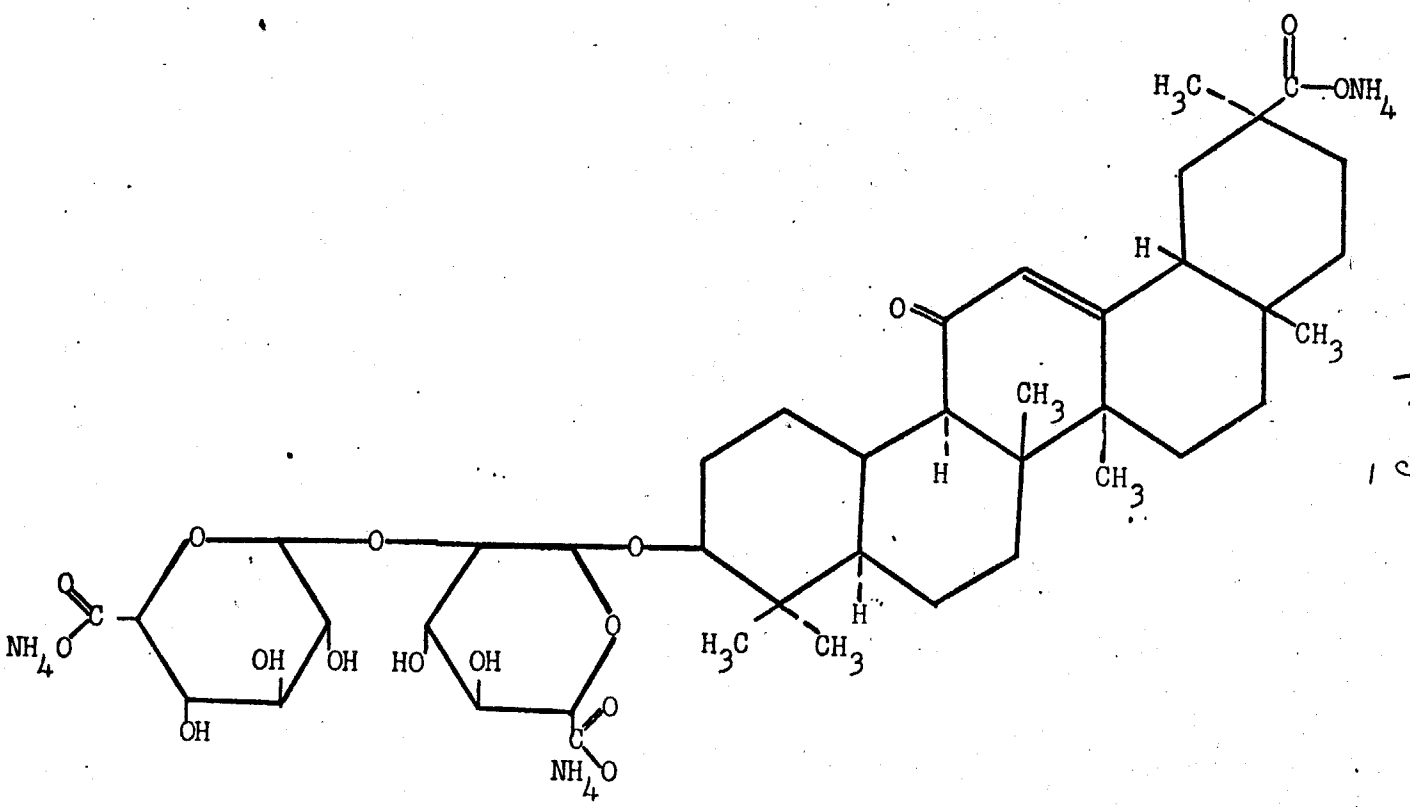
Glycyrrhizin, a glycoside consisting of glycyrrhetic acid and glucuronic acid as illustrated in Figure I, is prepared by a hot water extraction of licorice root (Nieman, 1957). Sulfuric acid precipitation and neutralization with ammonia yields ammoniated glycyrrhizin (AG), a commercially available product possessing the characteristic licorice flavor. AG is reported to be an effective natural sweetener, 100 times sweeter than sucrose in the presence of sucrose, and, therefore, has potential as a sugar substitute in low calorie foods and beverages. It also has the ability to intensify such flavors as chocolate, caramel, maple and root beer and could, therefore, be used in the manufacture of such products at lower costs (McAndrews and Forbes Co., 1956). It is widely used by the tobacco industry in tobacco casing liquor and less widely used as a curing agent in bacon, an emulsifier in ice cream, an oxidation inhibitor in chewing gum and a fat stabilizer in coating chocolate.

Initial reports on the development of clinical side effects with the usage of licorice extract, succus liquiritae, for the treatment of ulcers appeared when approximately 20% of the treated patients developed edema, headache and shortness of breath (Revers, 1948). These effects were readily reversible when treatment was terminated.

A scheme was designed for ascertaining the cause of edema in the human subject using the dried licorice extract which contains approximately 15% glycyrrhizin (Molhuysen et al., 1950). It was determined that the active principle of the licorice extract was similar in its metabolic activities to DOC, though the effects persisted for longer periods when treatment was terminated.

The electrolyte-active principle of licorice extract, ammonium glycyrrhizinate, was then prepared and orally administered to a group of normal subjects (Louis and Conn, 1956). Doses of 4 to 6 gm/day produced a great retention of sodium, chloride and water, a mild increase in urinary

Figure 1. Chemical structure of Ammoniated Glycyrrhizin.



→ C_{21}

Cuts mission ?

C_{30} = glycyrrhetinic acid

potassium, mild inhibition of endogenous ACTH production as indicated by a consistent decrease in excretion of 17-keto steroids and inhibition of pituitary release of melanocyte-stimulating hormone. It was concluded that ammonium glycyrrhizinate acted in the body in a way similar to an adrenal cortical steroid but whether this effect was inherent in the compound as administered or resulted from metabolic transformation within the body could not be determined.

Beginning about the mid-1960's, an increasing number of medical reports appeared in the literature concerning the development of a reversible hypertension accompanied by hypokalemia and hypernatremia apparently due to ingestion of licorice candy in amounts approximating 100 gm/day over a period of several months to several years (Conn et al., 1968; Gross et al., 1966; Koster and David, 1968; Lefebvre and Marc-Aurele, 1968; McNicholl and Kilroy, 1969; Pelner, 1969; Rausch-Stroomann, 1968; Vianna, 1971). This condition closely resembles aldosteronism but is characterized by a suppression of both plasma renin activity and aldosterone secretion and is reversible upon cessation of licorice ingestion (Conn et al., 1968).

The action of glycyrrhizin in patients with partial or total adrenal insufficiency is variable. A patient with Addison's disease who did not respond to ACTH also showed no response to licorice extract suggesting that the action of glycyrrhizin is mediated by the adrenals (Molhuysen et al., 1950). In three separate cases of patients who were bilaterally adrenalectomized an attempt was made to substitute glycyrrhizinic acid for cortisone therapy (Hudson et al., 1954). From this experiment it was concluded that glycyrrhizinic acid alone was insufficient for adequate maintainence of adrenalectomized patients. However, patients could be maintained on subminimal doses of cortisone with the addition of glycyrrhizinic acid. Purified ammonium glycyrrhizinate was unable to produce any electrolyte effects in two patients with congenital adrenal hyperplasia while a patient with Cushing's syndrome retained sodium

and chloride and demonstrated potassium loss as did the normal subjects in this study (Louis and Conn, 1956).

Additional data supporting the mineralocorticoid-like effects of licorice were provided when the effects of licorice on the renal excretion of sodium and potassium were successfully blocked with spironolactone, an aldosterone and DOC antagonist (Salassa et al., 1962).

The results of animal studies using licorice and its extracts also present some degree of variation. It had been shown that the aglycone, glycyrrhetic acid, had DOCA-like effects in both the normal and Addisonian human subject (Card et al., 1953). Adrenalectomized male rats treated with glycyrrhetic acid showed no decrease in sodium excretion nor any significant change in serum sodium or potassium at a dose of 3 mg/rat/day for a period of 5 days (Galal, 1955). Under similar conditions, DOCA at 1 mg/rat/day did produce the classic picture of sodium retention. Glycyrrhetic acid did produce inhibition of diuresis with a single 100 mg dose in normal unanesthetized rats and in diabetic (posterior pituitary removed) rats indicating that the pituitary is not involved in the drug action.

It was further suggested that the aglycone glycyrrhetic acid, because of its αβ-unsaturated ketonic group, might act by inhibition of the metabolism of the adrenocortical steroids (Atherden, 1958). In an in vitro study using rat liver homogenates, it was shown that glycyrrhetic acid was a powerful inhibitor of DOC and progesterone metabolism and from studies with the 25,000 x g supernatant of the rat liver dialyzed to remove citrate, cis-aconitate and isocitrate, it was concluded that this inhibition of progesterone metabolism was a direct effect on the hepatic steroid metabolizing system. It was further suggested that the 11-oxo group was necessary for the inhibition since 11-deoxyglycyrrhetic acid exhibited only minimal inhibition in the system.

Further studies in rats confirmed the anti-diuretic effect of glycyrrhetic

acid and also demonstrated sodium retention and urinary potassium excretion in the intact animal with a single dose of 125 mg/kg (Finney et al., 1958). In addition, glycyrrhetic acid had no glucocorticoid effects on adrenalectomized rats subjected to cold stress.

A comparison of the hypertensive effects of DOCA, licorice and AG in unilaterally nephrectomized male rats over a period of 60 days was made (Girerd et al., 1958). Doses of the three substances were as follows:: three 25 mg pellets of DOCA by interscapular implantation; 10 gm/kg/day of licorice and 1 gm/kg/day of AG by oral intubation. All three treatments produced a progressive rise in blood pressure with licorice causing the highest rise over the control levels. Control rats exhibited a moderate rise in blood pressure to a maximum of 125 mm Hg. In this study, saline intake, growth and survival time and organ weights were also followed. AG treated rats showed no difference in saline intake and had a normal growth pattern up to 36 days before they began to level off below control weights. Kidney and adrenal weights were higher in all three test groups and survival time of the AG treated rats was 33% below controls but above that for the other test groups. Upon autopsy, kidney and brain lesions were found in all three groups receiving treatment.

In a later study using these same criteria, the lack of an effect with AG at 1 gm/kg/day over a 48 day period on adrenalectomized male rats as measured by survival, saline intake and blood pressure was demonstrated (Girerd et al., 1960). This confirmed the previous study by Galal (1955) with glycyrrhetic acid showing that the effects of licorice and its extracts are not true mineralocorticoid but rather that they act through the adrenal gland.

The anti-diuretic effects in rats and rabbits was again confirmed using the sodium salt of glycyrrhizin at 100 mg/kg, oral and i. p. (Gujral et al., 1961b). However, this same drug at 25 mg/kg i. v. produced a slight hypotensive effect in anesthetized dogs. In a related study, glycyrrhizin at an

oral dose of 400 mg/kg failed to prolong the survival time of adrenalectomized male rats (Gujral et al., 1961a).

Glycyrrhizin has also been shown to have an inhibitory effect on the following biological actions of cortisone: deposition of liver glycogen in adrenalectomized rats treated with cortisone; induction of tryptophan pyrrolase; and increased cholesterol biosynthesis with cortisone treatment (Kumagai et al., 1966a, 1966b, 1967). Glycyrrhizin also inhibited the effects of estradiol-17β on the uterine weight and β-glucuronidase activity at specific dose ratios. The mechanism of these inhibitory effects of glycyrrhizin remains unknown.

In view of the apparent action of glycyrrhizin on blood pressure and electrolytes in humans and animals and the failure of similar responses in adrenalectomized animals, it appears likely that the action of glycyrrhizin is in some way mediated by the adrenal gland. It is not known, however, what the mechanism of this apparent mediation might be.

It is the purpose of this study to determine if AG acts in the intact animal by inhibition of the metabolic conversion of DOC to corticosterone and its subsequent products. An inhibition at this point would result in an increase in DOC and production of hypertension, sodium and water retention and potassium loss. Furthermore, such inhibition would also account for the decreased levels of aldosterone found in the human subject.

The work presented in this thesis includes the measurement of blood pressure in three strains of rats, determination of serum electrolytes and plasma and adrenal corticosterone, conversion of pregnenalone to DOC and corticosterone by the adrenal gland, and comparison of the organ weight-body weight ratios in intact rats treated with the technical grade of AG.

CHAPTER II

MATERIALS AND METHODS

Chemicals and Reagents

Animals were treated either with the purified mono-ammonium glycyrrhizinate or ammoniated glycyrrhizin (technical grade) shown to contain 38% mono-ammonium glycyrrhizinate by GLC analysis (Larry et al., 1970). Both compounds were purchased from McAndrews and Forbes Company, Camden, New Jersey.

Radioactive labeled pregnenalone-7-^{3}H (0.25 mCi/0.25 ml) was purchased from New England Nuclear Corporation, Boston, Massachusetts.

Deoxycorticosterone, pregnenalone and corticosterone were purchased from Sigma Chemical Company, St. Louis, Missouri. Injectable corticotropin was purchased from Armour Pharmaceutical Company, Chicago, Illinois.

Chemicals used for steroid assays were Certified Reagent grade excepting methanol which was Spectrograde. The chloroform used for spectrofluorescent analysis was specifically tested before use for low fluorescent background.

Other Materials

Blood pressure determinations were done using a PE-300 Programmed Electrosphygmomanometer purchased from Narco Biosystems, Houston, Texas. Fluorescence was measured on the Aminco Fluorimeter and the Beckman LS-255 Liquid Scintillation System was used for determining all radioactivity. Serum electrolytes were measured on an IL-343 Flame Photometer with automatic dilutor. Thin layer chromatography was carried out on pre-coated, plastic-backed plates purchased from Baker Chemical Company.

Animal Experiments

Osborne-Mendel rats were obtained from the in-house breeding colony at the Food and Drug Administration, Washington, D. C. Sprague-Dawley rats were purchased from Blue Spruce Farms, Alamont, New York. Specially bred spontaneously hypertensive rats from the Wistar strain were obtained from the breeding colony at the Washington Hospital Center, Washington, D. C.

Subacute oral dosing of the animals was accomplished by administering an aqueous solution of the test compound by stomach tube on a mg/kg body weight/day basis. Control animals in these studies received equivalent amounts of water by stomach tube. These animals were given 0.9% sodium chloride as drinking fluid and were maintained on a diet of Purina Laboratory Chow. Both saline and chow were available ad lib.

Animals on the feeding study were given a diet consisting of 4% technical grade AG mixed in ground Purina Laboratory Chow. Diet and saline were allowed ad lib and consumption was measured on a weekly basis.

Blood Pressure Determination 108°F!

Animals were heated at 42°C for 15 minutes prior to measuring the blood pressure by the tail cuff method. The animal was placed in a wire mesh holder of suitable size and placed under a heating lamp adjusted to maintain a temperature of approximately 42°C. A tail cuff was placed at the base of the tail and a Korotkoff sound microphone was attached directly below the pressure cuff by means of a rubberized band and a clamp. Both the microphone and the tail cuff were attached to the PE-300 Programmed Electrosphygmomanometer which was adjusted to automatically inflate and deflate the cuff at a pre-set rate to a maximum of 250 mm Hg. An inflation-deflation rate of 25 mm Hg/second was maintained. This cycle was repeated approximately every 20 seconds. In order to adapt the animal to the blood pressure measurement, several cycles, never less than three, were run before the blood pressure was recorded on the Sanborn Polygraph Recorder. Average blood pressure was determined from the three most stable consecutive cycles.

Sacrifice of Animals

The orally dosed animals were anesthetized with ether approximately 30 minutes after s. c. injection with 5 units of corticotropin. When the animal

had reached the surgical stage of anesthesia, the chest cavity was opened and blood was rapidly removed by heart puncture. A portion of the whole blood was mixed with powdered heparin for plasma corticosterone assay and the remainder was retained for serum electrolyte determinations. The adrenals were rapidly removed and placed in a petri dish on ice, trimmed of all visible fat, weighed and quartered in preparation for corticosterone analysis. The liver, heart, spleen, kidneys, testes and brain were removed, cleaned of all visible fat and weighed before being discarded.

The subacutely fed animals were sacrificed in the same manner except they were not injected with corticotropin prior to sacrifice and all blood removed was heparinized for corticosterone assay.

Adrenal Incubation

The cleaned and quartered adrenals were placed in a 25 ml Erlenmeyer flask containing 3 ml of Krebs-Ringer-Bicarbonate media to which was added 0.2% glucose and 0.5% Bovine serum albumin immediately prior to use. The flasks were preincubated in a Dubonoff Shaking Bath for 1 hour at 37°C, 60-100 cycles/minute under a mixture of O_2-CO_2 (95%-5%). At the end of 1 hour, the incubation media was removed with a glass disposable pipette and stored in a screw-top tube at -4°C for steroid analysis. This incubation media was labeled Incubation A.

A second incubation was carried out under the same conditions in the presence of 2.7 ml of the KRBG-A and 0.3 ml KRBG-A containing 5×10^{-2} mM DOC. This incubation media was labeled Incubation B.

In experiment six using the S. D. strain, the preincubation step was omitted. Instead, the adrenals were incubated in 4 ml of the KRBG-A to which was added 0.1 ml of pregnenalone-7-^{3}H (1 μC/0.1 ml in methanol).

Corticosterone Extraction and Analysis

Extraction and analysis were done by the method of Zenker and Bernstein (1958). A 0.5 ml portion of each of the incubation media or the undiluted plasma was extracted by shaking for 15 minutes in 10 ml of fluorescent grade chloroform. The water layer was then aspirated and discarded. Five ml of the remaining chloroform layer was added to 3 ml of Fluorescent Reagent which contained 50 parts ethanol, 50 parts water and 240 parts concentrated sulfuric acid and the mixture was agitated for 15 minutes. The two phases were allowed to separate and the upper chloroform phase was aspirated and discarded. The lower acid phase was measured for fluorescence in an Aminco fluorimeter approximately 45 minutes after the second extraction. Corticosterone was expressed in terms of μg/mg adrenal or μg/100 ml plasma as determined by a standard curve.

Serum Electrolytes

The whole blood removed during autopsy was allowed to clot and the serum layer was removed according to standard methods. The undiluted serum was then automatically aspirated into the IL-343 Flame Photometer where it was automatically diluted with a lithium standard to give a digital readout expressed in mEq/liter.

Corticosteroid Extraction

The measured volume of the adrenal incubation media remaining after corticosterone assay was mixed with 10 volumes of dichloromethane per volume of incubate. Extraction was carried out by shaking the dichloromethane-incubate mixture in a separatory funnel for 15 minutes after which the two phases were allowed to separate for approximately 10 minutes. The lower dichloromethane layer was removed into a test tube and evaporated to dryness under a stream of nitrogen in a 60°C water bath. The sides of the tube were

washed down three times with 0.3 ml aliquots of dichloromethane and evaporated to dryness after each wash. The residue was stored in a refrigerator for later TLC separation.

Thin Layer Chromatography

Chromatography of the extracted corticosteroids was carried out in a two directional system using plastic-backed, pre-coated Bakerflex IB-F, 20 x 20 cm plates of Silica Gel containing fluorescein. The solvent system used for both directions of development was a mixture of chloroform-methanol (97:3) prepared fresh before each use. The tanks were allowed to equilibrate for 30 minutes prior to use. The plates were activated for 10 minutes at 90°C before spotting. The dry extract was taken up in three 50 lambda portions of dichloromethane containing 50 μg of cold DOC and 25 μg of cold corticosterone for purposes of easier detection of the fluorescent spot. The plate was again activated for 10 minutes and placed in the solvent system until the solvent front had reached 150 mm from the point of application. Before chromatography was undertaken in the second direction, the tanks were filled with fresh solution and equilibrated for 30 minutes and the plates were activated for 10 minutes. The plate was then turned 90° and placed in the tank for chromatography as above.

Because of the low concentration and high volatility of methanol with respect to the chloroform, it was important to take precautions in the use of this system. The solution was prepared fresh daily and changed after each use in order to minimize the evaporation of the methanol which would result in incomplete separation of the pregnenalone and DOC. In addition, opening of the equilibrated tanks was held to a minimum and only one plate was developed per tank.

Corticosterone and DOC were visualized by UV fluorescence after which

the plate was sprayed with a solution of o-phosphoric acid and water (1:1) and heated for 5 minutes at 90°C for visualization of the pregnenalone which appeared as a bluish-pink spot.

Measurement of Radioactivity

Those portions of the TLC plate containing the labelled pregnenalone, DOC and corticosterone were scraped into separate liquid scintillation vials, the acid was neutralized with a drop of concentrated ammonium hydroxide and 10 ml of Bray's scintillation fluid was added. The scintillation fluid consisted of a mixture of dioxane:methanol (12.4:1.5) containing naphthalene (6.47%) and Omniflor (New England Nuclear, 0.43%). Results are expressed as a simple ratio of corticosterone to DOC (DPM).

CHAPTER III

RESULTS

Preliminary Dose Ranges and Regimens in the Osborne-Mendel Rat

The first experiments were undertaken in an attempt to find an effective dose to produce hypertension and an appropriate means of dosing the animals. Feeding of a 1% diet of tech-AG to five female O. M. rats weighing an average of 250 gm at the start for a period of 17 weeks failed to produce any changes in blood pressure or corticosterone levels over control fed animals.

A second group of five female O. M. rats weighing 150 gm at the start of the experiment were placed on the following dosage schedule using a 1% solution of pure-AG administered by oral intubation:

Weeks 1-3 - 100 mg/kg/day

Week 4 - 200 mg/kg/day

Weeks 5-8 - 300 mg/kg/day

At the end of the eight week dosing period, there were no changes in blood pressure nor plasma and adrenal corticosterone levels over those of control rats.

In the event that the female rat might be unresponsive to this agent, a third group of ten O. M. rats, male, weighing an average of 135 gm were divided into two groups of five each, one group receiving a daily oral dose of 500 mg/kg pure-AG and the other receiving 1000 mg/kg/day. Ten rats of the same sex and age were dosed with an equivalent amount of water. At the end of 30 days of dosing, no significant changes were found in blood pressure, serum electrolytes nor plasma and adrenal corticosterone levels. Insolubility of the pure-AG at a concentration greater than 1% in water precluded any attempt at increasing the dosage in these animals.

Comparison of Sprague-Dawley and Osborne-Mendel Strains When Dosed With Ammoniated Glycyrrhizin

Because of the failure to produce an increase in blood pressure in

these first three experiments, it was decided to repeat the oral dosing studies with tech-AG, the commercially available and more soluble substance, using another strain of rats known to respond to hypertensive agents such as DOCA and saline. For the purpose of this study, the S. D. strain of rats was selected. To rule out any differences in response by the two strains to the tech-AG as compared to the pure-AG, the O. M. strain, which was apparently unresponsive to the pure-AG, was also dosed with the tech-AG. All animals were allowed saline ad lib to insure an adequate salt intake.

Twenty male rats of each strain were divided into a test and control group of ten each. These groups were further divided so that five animals received a daily dose of 1000 mg/kg and the remaining five received a daily dose of 2000 mg/kg. Each of the control groups received an equivalent amount of water. All dosing was done by oral intubation and saline and food were allowed ad lib. The blood pressures were monitored on a weekly basis.

Figure 2 shows the blood pressure pattern for the S. D. rats. At the end of 2 weeks of treatment, the S. D. rats treated at 1000 mg/kg/day showed a small but significant increase in blood pressure which continued to show a slow rise over the remaining 4 weeks of treatment to reach a final level of 160 mm Hg. Those rats treated at 2000 mg/kg/day showed a significant increase in blood pressure by 3 weeks of treatment and it remained significantly increased until the end of 5 weeks, when the blood pressure showed a reversal of the steady rise and the animals were sacrificed due to their poor condition.

By way of contrast, the identically treated O. M. rats showed no consistant changes in blood pressure, as indicated in Figure 3. The statistically significant increase in blood pressure in those animals on the 1000 mg/kg/day level seen at week 5 is probably not of biological significance. The experiment was terminated after 5 weeks due to poor condition of the animals.

Figure 2. Blood pressure patterns of male Sprague-Dawley rats treated daily by oral intubation with tech-AG. Each point represents the average blood pressure of 5 test or 10 control animals, except where otherwise noted by (n), on the figure ± SEM.

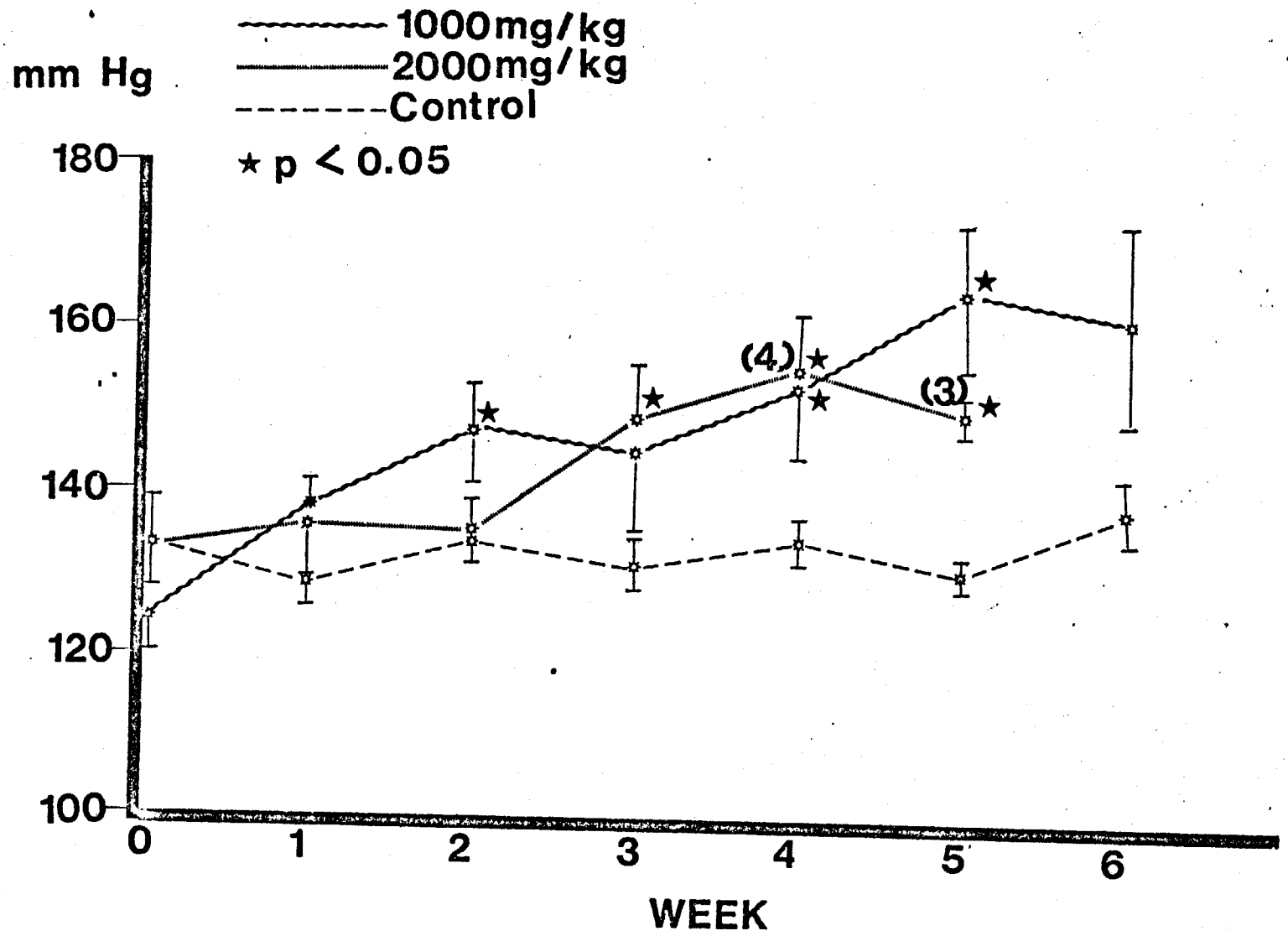
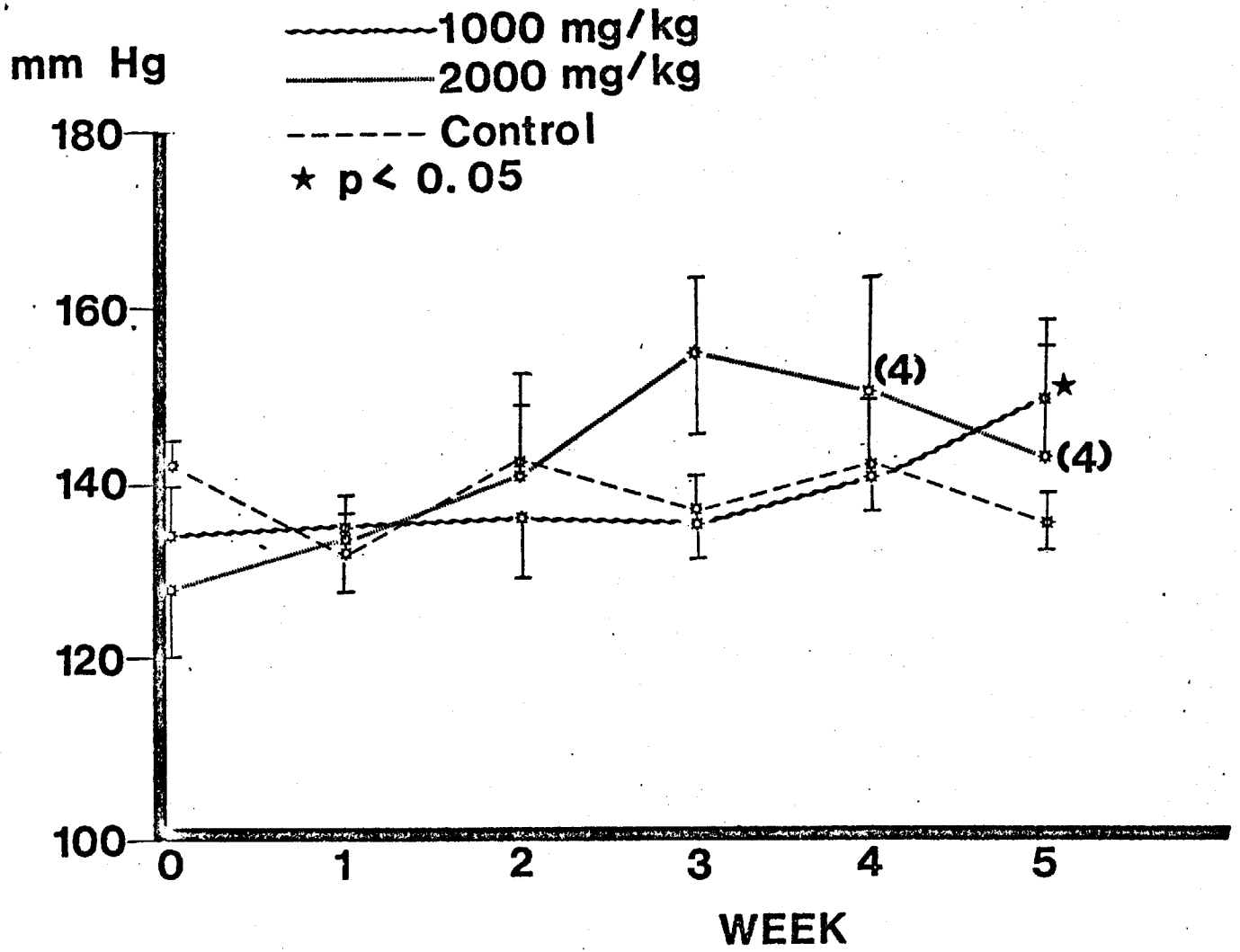

1000mg/kg
2000mg/kg
Control
★ p < 0.05
mm Hg
180
160
140
120
100
0
1
2
3
4
5
6
WEEK
(4)
(3)

Figure 3. Blood pressure patterns of male Osborne-Mendel rats treated daily by oral intubation with tech-AG. Each point represents the average blood pressure of 5 test or 9 control animals, except where otherwise noted by (n), on the figure ± SEM.


1000 mg/kg
2000 mg/kg
Control
★ p< 0.05
mm Hg
180
160
140
120
100
0
1
2
3
4
5
WEEK
(4)
(4)
★

Serum electrolytes and plasma and adrenal corticosterones for S. D. and O. M. rats are shown in Tables 1 and 2, respectively. At termination of the experiment, serum electrolytes were determined. Neither strain of rats showed any significant changes; however, a tendency toward hypokalemia was evident in the O. M. strain. The S. D. rats treated at 1000 mg/kg/day showed a significant decrease in plasma corticosterone and in the in vitro adrenal corticosterone level of Incubation B. In contrast, the S. D. rats treated at the higher level showed a significant increase in plasma corticosterone and no change in the in vitro adrenal levels of the steroid. In the O. M. rats, neither dosage level produced any significant changes in plasma or adrenal corticosterone.

Table 3 shows the organ weight-body weight ratios of the S. D. rats. Little change occurred in those rats dosed at 1000 mg/kg/day with the exception of a significant decrease in testes and brain weight-body weight ratios. On the other hand, all organ weights at the higher level, excepting the adrenal weights, showed a tendency to increase with the heart weight-body weight ratio increasing significantly over control weights.

As shown in Table 4, the O. M. rats treated at the lower dosage level showed a significantly decreased spleen weight-body weight ratio while those on the higher dosage showed a significant increase in kidney, heart and testes weight-body weight ratios.

At the end of the treatment schedule, there were no significant differences in body weights at either treatment level in either strain despite the poor condition of both strains at the higher dosage level. This is shown in Table 5 for the S. D. rats and in Table 6 for the O. M. rats.

Daily Oral Dosing of Spontaneously Hypertensive Rats with Tech-AG

In order to evaluate the effects of a hypertensive agent in those strains showing a tendency toward hypertension, it was decided to dose a

Table 1

SERUM ELECTROLYTES, PLASMA CORTICOSTERONE AND IN VITRO ADRENAL CORTICOSTERONE OF MALE SPRAGUE-DAWLEY RATS TREATED DAILY BY ORAL INTUBATION WITH TECH-AG

Treatment	Electrolytes[1]		Corticosterone[2]		
			µg/ 100 ml	µg/ mg Adrenal	
	Na^+	K^+	Plasma	A[3]	B[4]
1000 mg/kg (5)[5] 6 weeks	148 ± 1	4.9 ± 0.4	34* ± 1.7	0.13 ± 0.01	0.16* ± 0.02
Control (5) 6 weeks	149 ± 1	5.0 ± 0.1	40 ± 0.5	0.16 ± 0.01	0.22 ± 0.01
2000 mg/kg (3) 5 weeks	167[6] ± 0	6.7[6] ± 0.0	37* ± 1.8	0.18 ± 0.01	0.26 ± 0.01
Control (5) 5 weeks	152 ± 3	6.1 ± 0.8	30 ± 0.5	0.14 ± 0.02	0.26 ± 0.01

[1] mEq/l ± SEM; [2] µg ± SEM; [3] preincubation as described in methods; [4] incubation with DOC as described in methods; [5] (n) = number of animals at the end of the experiment; [6] represents value for one animal only; * statistically significant $p < 0.05$.

Table 2

SERUM ELECTROLYTES, PLASMA CORTICOSTERONE AND IN VITRO ADRENAL CORTICOSTERONE OF MALE OSBORNE-MENDEL RATS TREATED DAILY BY ORAL INTUBATION WITH TECH-AG FOR FIVE WEEKS

Treatment	Electrolytes[1]		Corticosterone[2]		
			µg/100 ml	µg/mg Adrenal	
	Na^+	K^+	Plasma	A[3]	B[4]
1000 mg/kg (5)[5]	144 ± 1	4.7 ± 0.3	27 ± 3.4	0.13 ± 0.02	0.22 ± 0.004
Control (5)	145 ± 1	5.3 ± 0.3	26 ± 2.3	0.13 ± 0.01	0.22 ± 0.01
2000 mg/kg (4)	149 ± 1	5.0 ± 0.4	38 ± 1.2	0.17 ± 0.02	0.17 ± 0.01
Control (4)	150 ± 3	5.9 ± 0.6	33 ± 2.3	0.14 ± 0.01	0.15 ± 0.02

[1] mEq/l ± SEM; [2] µg ± SEM; [3] preincubation as described in methods; [4] incubation with DOC as described in methods; [5] (n) = number of animals at the end of the experiemnt.

Table 3

ORGAN WEIGHT-BODY WEIGHT RATIOS OF MALE SPRAGUE-DAWLEY RATS TREATED DAILY BY ORAL INTUBATION WITH TECH-AG

Treatment	Liver[1]	Kidney[1]	Spleen[1]	Heart[1]	Testes[1]	Brain[1]	Adrenal[2]
1000 mg/kg (5)[3] 6 weeks	3.8 ± 0.2	0.72 ± 0.02	0.17 ± 0.02	0.31 ± 0.01	0.86* ± 0.05	0.45* ± 0.01	12.9 ± 0.8
Control (5) 6 weeks	3.7 ± 0.1	0.69 ± 0.02	0.18 ± 0.00	0.32 ± 0.01	1.01 ± 0.02	0.50 ± 0.01	11.9 ± 0.5
2000 mg/kg (3) 5 weeks	4.2 ± 0.2	0.86 ± 0.07	0.18 ± 0.03	0.36* ± 0.03	1.13 ± 0.03	0.58 ± 0.02	14.3 ± 1.2
Control (5) 5 weeks	3.6 ± 0.2	0.75 ± 0.06	0.16 ± 0.01	0.28 ± 0.00	0.92 ± 0.07	0.50 ± 0.05	14.4 ± 0.9

[1] gm tissue/ 100 gm final body weight ± SEM; [2] mg tissue/100 gm final body weight ± SEM; [3] (n) = number of animals at the end of the experiment; * statistically significant $p < 0.05$.

Table 4

ORGAN WEIGHT-BODY WEIGHT RATIOS OF MALE OSBORNE-MENDEL RATS TREATED DAILY BY ORAL INTUBATION WITH TECH-AG FOR FIVE WEEKS

Treatment	Liver[1]	Kidney[1]	Spleen[1]	Heart[1]	Testes[1]	Brain[1]	Adrenal[2]
1000 mg/kg (5)[3]	3.2 ± 0.2	0.75 ± 0.03	0.13* ± 0.01	0.28 ± 0.01	0.93 ± 0.04	0.38 ± 0.02	11.3 ± 0.7
Control (5)	3.2 ± 0.1	0.68 ± 0.02	0.16 ± 0.01	0.30 ± 0.01	0.88 ± 0.04	0.32 ± 0.03	11.0 ± 1.0
2000 mg/kg (4)	4.0 ± 0.2	0.83* ± 0.02	0.16 ± 0.02	0.36* ± 0.01	1.03* ± 0.01	0.46 ± 0.01	12.6 ± 1.3
Control (4)	3.6 ± 0.1	0.75 ± 0.01	0.19 ± 0.01	0.32 ± 0.01	0.94 ± 0.03	0.42 ± 0.02	12.8 ± 0.9

[1] gm tissue/100 gm final body weight ± SEM; [2] mg tissue/100 gm final body weight ± SEM; [3] (n) = number of animals at the end of the experiment; * statistically significant $p < 0.05$.

Table 5

GROWTH ANALYSIS OF MALE SPRAGUE-DAWLEY RATS TREATED DAILY BY ORAL INTUBATION WITH TECH-AG

Treatment	Initial Weight (I) grams ± SEM	Final Weight (F) grams ± SEM	% Change $\frac{F - I}{I}$
1000 mg/kg (5)[1] 6 weeks	271 ± 8	392 ± 15	45%
Control (5) 6 weeks	238 ± 18	355 ± 9	49%
2000 mg/kg (3) 5 weeks	237 ± 16	335 ± 15	41%
Control (5) 5 weeks	287 ± 15	369 ± 33	28%

[1] (n) = number of animals at the end of the experiment.

Table 6

GROWTH ANALYSIS OF MALE OSBORNE-MENDEL RATS TREATED DAILY BY ORAL INTUBATION WITH TECH-AG FOR FIVE WEEKS

Treatment	Initial Weight (I) grams ± SEM	Final Weight (F) grams ± SEM	% Change $\frac{F - I}{I}$
1000 mg/kg (5)[1]	390 ± 8	450 ± 16	15%
Control (5)	395 ± 10	466 ± 16	18%
2000 mg/kg (4)	363 ± 17	415 ± 21	14%
Control (4)	404 ± 29	455 ± 28	13%

[1] (n) = number of animals at the end of the experiment.

strain of spontaneously hypertensive rats with tech-AG. The 1000 mg/kg/day dose was selected as it was better tolerated with less signs of toxicity while still producing the hypertensive effect. Both sexes were dosed in order to rule out the possibility of sex-related differences.

Twelve male and twelve female Wistar S. H. rats weighing an average of 300 and 185 gm, respectively, were equally divided into test and control groups. All animals were allowed food and saline ad lib. Initial blood pressures of all animals in this study, test and control alike, were considerably higher than the initial readings for the O. M. and S. D. strains previously tested, with the male rats averaging 175 mm Hg and the females 155 mm Hg.

Figure 4 illustrates the blood pressure pattern of the male rats. Control rats remained relatively stable around 175 mm Hg while the test group showed a significant increase after 1 week of treatment and continued in a general rise throughout the 9 weeks treatment to a high of 240 mm Hg. The somewhat erratic results and lack of statistical significance over the last 3 weeks were due to poor condition of the animals resulting in three deaths. In addition, as the blood pressure levels passed 200 mm Hg, readings became more variable and the animals more hyperactive making consistent readings difficult to attain.

Figure 5 shows the blood pressure pattern of the female rats. A rise in blood pressure became evident after 4 weeks of treatment reaching a significantly high level at 6 weeks and leveling off at 7 weeks around 220 mm Hg. Control females remained relatively stable around 155 mm Hg.

Table 7 illustrates the levels of serum electrolytes, plasma corticosterone and in vitro adrenal corticosterone for both sexes. Treated male rats showed a very small but significant increase in serum sodium. All of the S. H. rats had levels of potassium below that normally found in the rat, 3.1 mEq/l as compared to 5.5 mEq/l, however, there were no significant differences

Figure 4. Blood pressure pattern of male Wistar Spontaneously Hypertensive rats treated daily by oral intubation with tech-AG. N = 6 ± SEM except where otherwise noted.

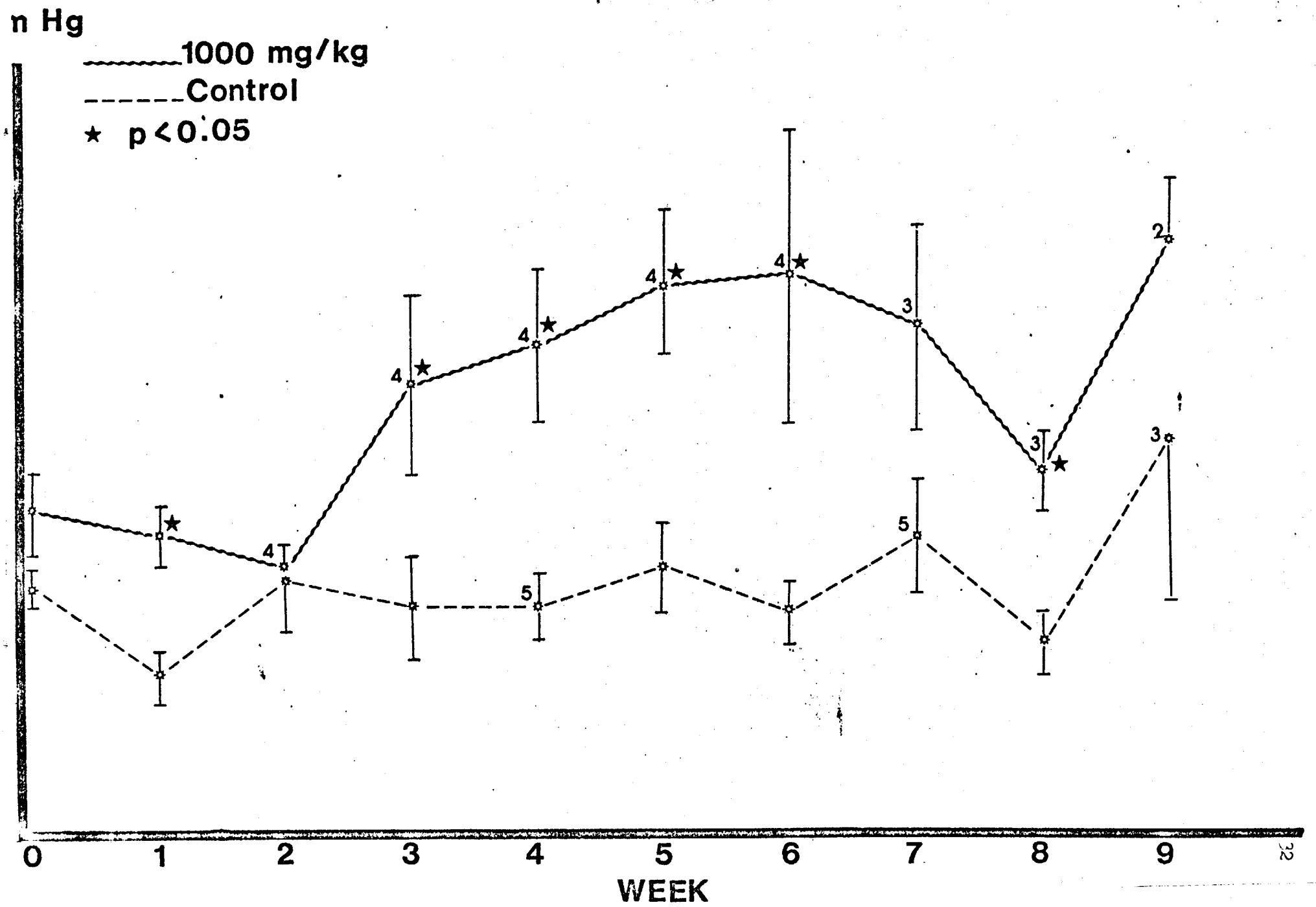

n Hg
1000 mg/kg
Control
★ p<0.05
4
4
4
4
4
3
3
2
4
5
5
3
0
1
2
3
4
5
6
7
8
9
WEEK

Figure 5. Blood pressure patterns of female Wistar Spontaneously Hypertensive rats treated daily by oral intubation with tech-AG. N = 6 ± SEM except where otherwise noted on the figure.

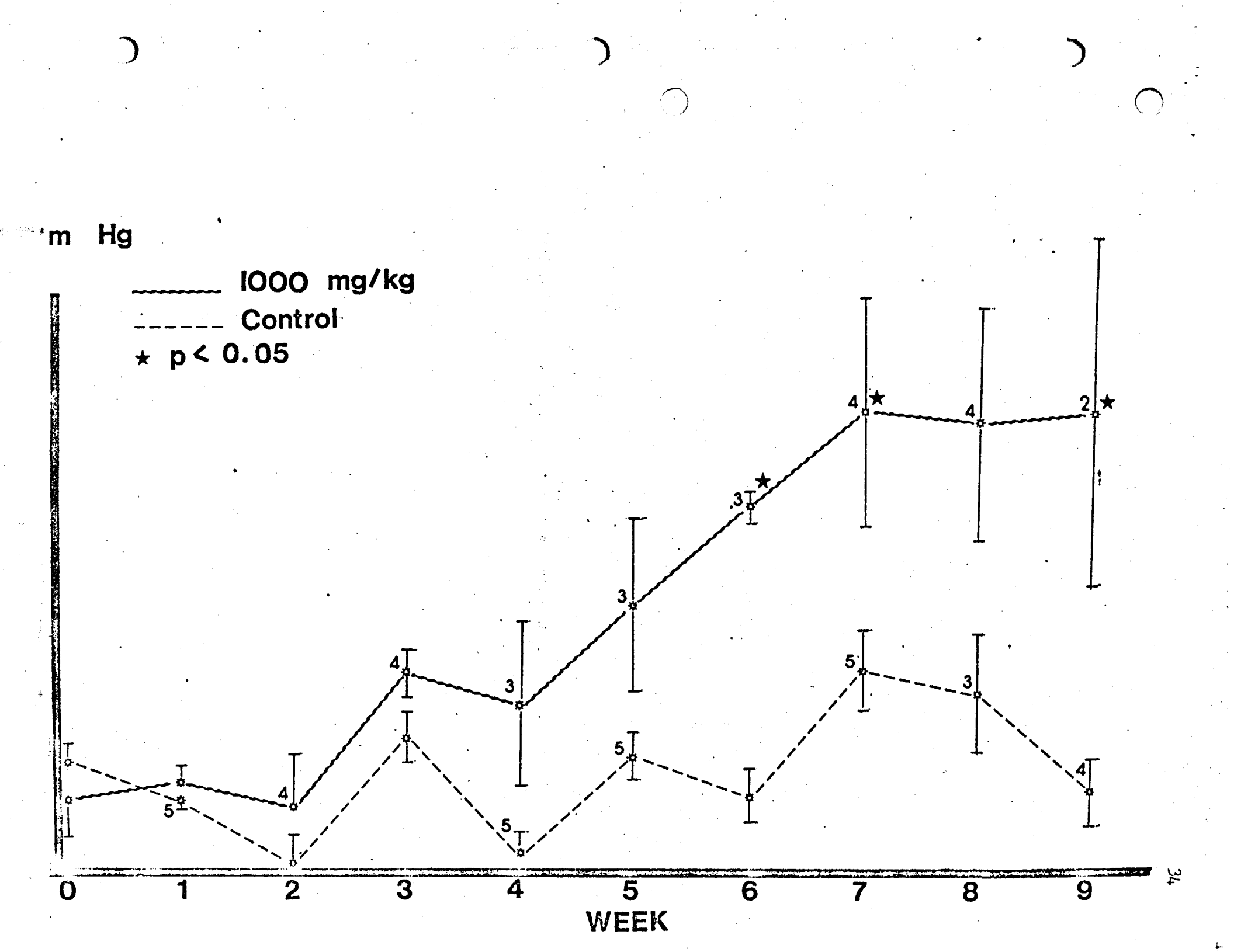

m Hg
1000 mg/kg
Control
★ p < 0.05
0
1
2
3
4
5
6
7
8
9
WEEK

Table 7

SERUM ELECTROLYTES, PLASMA CORTICOSTERONE AND IN VITRO ADRENAL CORTICOSTERONE OF MALE AND FEMALE WISTAR SPONTANEOUSLY HYPERTENSIVE RATS TREATED DAILY BY ORAL INTUBATION WITH TECH-AG FOR NINE WEEKS

Treatment	Electrolytes[1]		Corticosterone[2]		
			µg/ 100 ml	µg/ mg Adrenal	
	Na^+	K^+	Plasma	A[3]	B[4]
1000 mg/kg (3)[5] ♂	147* ± 1	3.0 ± 0.03	85 ± 9.8	0.20 ± 0.01	0.06 ± 0.02
Control (5) ♂	145 ± 0.4	3.2 ± 0.1	94 ± 1.0	0.21 ± 0.01	0.06 ± 0.003
1000 mg/kg (3) ♀	146 ± 0.6	3.2 ± 0.2	79* ± 10	0.21 ± 0.01	0.06 ± 0.01
Control (5) ♀	145 ± 1.1	3.1 ± 0.1	115 ± 2.0	0.19 ± 0.01	0.07 ± 0.01

[1] mEq/l ± SEM; [2] µg ± SEM; [3] preincubation as described in methods; [4] incubation with DOC as described in methods; [5] (n) = number of animals at the end of the experiment; * statistically significant $p < 0.05$.

between test and control rats. Plasma corticosterone levels were lower in both treated groups but were significantly lower only in the treated females. Both groups showed a high degree of variability, as can be seen by the SEM, when compared to control animals. There were no changes in in vitro adrenal corticosterone levels.

Table 8 shows the results of TLC separation of tritiated adrenal steroids after incubation of the adrenals in the presence of pregnenalone-7-^{3}H. The results are expressed as a simple ratio of DPM recovered. The corticosterone/deoxycorticosterone (C/D) ratio of the treated female rats was significantly higher than control levels. No other changes were found.

As seen in Table 9, all organ weight-body weight ratios of the treated female rats were significantly increased while the kidney, heart and adrenal weight-body weight ratios were significantly increased in the treated male rats.

Table 10 shows that the treated females failed to gain weight on the dosing schedule while the male rats remained unaffected by the treatment.

Subacute Feeding of Tech-AG for Six Weeks to Male Sprague-Dawley Rats

Because of the failure to obtain consistent effects on plasma and in vitro adrenal corticosterone levels, it was decided to feed a group of rats over a period of 6 weeks with the tech-AG and to serially sacrifice groups at various periods throughout the study to determine if a pattern for the corticosterone changes could be established. Forty male S. D. rats weighing 200 gm at the start were divided equally into test and control groups. The test group was placed on a diet of ground Purina Chow containing 4% tech-AG. The control group received plain chow. Both groups were allowed saline ad lib. Food and saline consumption were measured on a weekly basis.

Figure 6 shows the blood pressure pattern and saline intake over the

Table 8

RATIOS OF DPM RECOVERED FROM INCUBATION OF ADRENALS OF MALE AND FEMALE WISTAR SPONTANEOUSLY HYPERTENSIVE RATS IN THE PRESENCE OF PREGNENALONE-7-^{3}H AFTER TREATMENT BY DAILY ORAL INTUBATION WITH TECH-AG FOR NINE WEEKS

Treatment	C/D ± SEM	C/P ± SEM	D/P ± SEM
1000 mg/kg (3)[1] ♂	0.85 ± 0.21	0.081 ± 0.034	0.088 ± 0.016
Control (5) ♂	0.88 ± 0.08	0.060 ± 0.010	0.072 ± 0.016
1000 mg/kg (3) ♀	1.99* ± 0.29	0.151 ± 0.048	0.071 ± 0.016
Control (5) ♀	0.89 ± 0.09	0.048 ± 0.006	0.055 ± 0.007

[1] (n) = number of animals at the end of the experiemnt;
* statistically significant $p < 0.05$; C = corticosterone, D = deoxycorticosterone, P = pregnenalone.

Table 9

ORGAN WEIGHT-BODY WEIGHT RATIOS OF MALE AND FEMALE WISTAR SPONTANEOUSLY HYPERTENSIVE RATS TREATED DAILY BY ORAL INTUBATION WITH TECH-AG FOR NINE WEEKS

Treatment	Liver[1]	Kidney[1]	Spleen[1]	Heart[1]	Testes[1]	Brain[1]	Adrenal[2]
1000 mg/kg (3) ♂	3.9 ± 0.4	0.97* ± 0.04	0.22 ± 0.01	0.49* ± 0.02	1.03 ± 0.00	0.51 ± 0.02	14.0* ± 1.2
Control (5) ♂	3.8 ± 0.1	0.82 ± 0.02	0.20 ± 0.01	0.44 ± 0.00	0.95 ± 0.04	0.51 ± 0.03	11.4 ± 0.4
1000 mg/kg (3) ♀	5.8* ± 0.2	1.27* ± 0.06	0.54* ± 0.06	0.73* ± 0.05	---	1.02* ± 0.04	34.4* ± 2.0
Control (5) ♀	4.1 ± 0.1	0.78 ± 0.03	0.23 ± 0.02	0.46 ± 0.01	---	0.73 ± 0.03	21.5 ± 0.6

[1] gm tissue/100 gm final body weight ± SEM; [2] mg tissue/100 gm final body weight + SEM; [3] (n) = number of animals at the end of the experiment; * statistically significant $p < 0.05$.

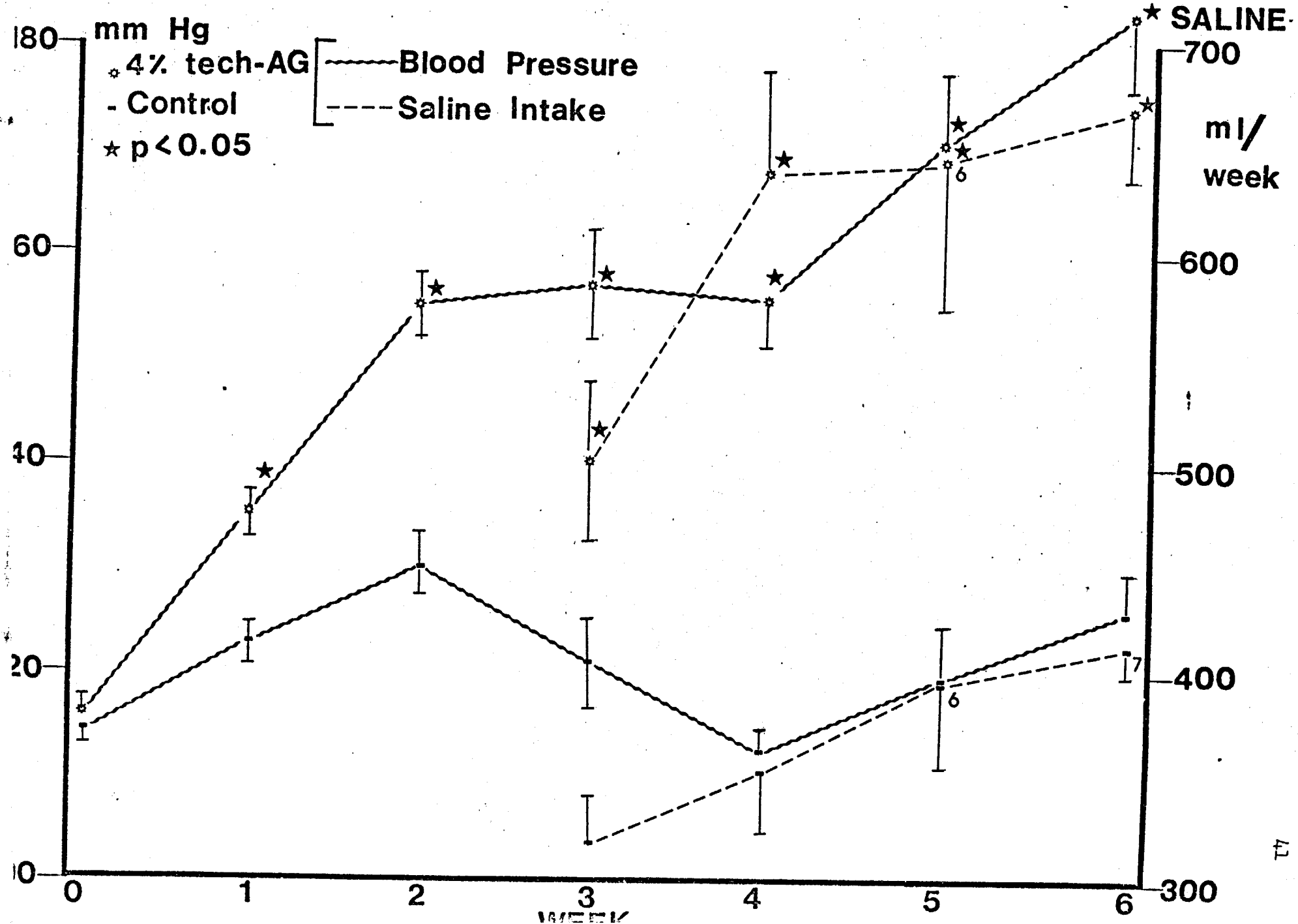
Table 10

GROWTH ANALYSIS OF MALE AND FEMALE WISTAR SPONTANEOUSLY HYPERTENSIVE RATS TREATED DAILY BY ORAL INTUBATION WITH TECH-AG FOR NINE WEEKS

Treatment	Initial Weight (I) grams ± SEM	Final Weight (F) grams ± SEM	% Change $\frac{F - I}{I}$
1000 mg/kg (3)[1] ♂	294 ± 7	323 ± 7	10%
Control (5) ♂	304 ± 10	340 ± 11	12%
1000 mg/kg (3) ♀	187 ± 5	173* ± 6	- 7%*
Control (5) ♀	188 ± 4	210 ± 2	12%

[1] (n) = number of animals at the end of the experiment; * statistically significant $p < 0.05$.

Figure 6. Blood pressure patterns and saline intake of male Sprague-Dawley rats fed subacutely a diet of ground Purina Chow containing 4% tech-AG. Each point represents an average for the following numbers of animals: weeks 0 and 1, n = 20; week 2, n = 16; week 3, n = 12; weeks 4, 5 and 6, n = 8 except where otherwise noted on the figure.


mm Hg
4% tech-AG
- Control
★ p<0.05
Blood Pressure
Saline Intake
★ SALINE
ml/
week
180
60
40
20
0
700
600
500
400
300
0
1
2
3
4
5
6
WEEK

6 week period. A significant increase in blood pressure was reached after 1 week on the diet and continued a steady rise throughout to a high of 185 mm Hg as compared to 125 mm Hg for the control animals. Saline intake, beginning at 3 weeks, paralleled the rise in blood pressure indicating an increased saline appetite in the treated animals.

Figure 7 shows a comparison between food consumption and weight gain for both groups. Intake and weight gain were parallel throughout the experiment with the treated group, in general, somewhat higher than controls. However, no significant differences were found.

Table 11 shows the plasma and in vitro adrenal corticosterone levels at various times during the experiment. A slightly higher plasma corticosterone was evident after 1 week of treatment and it continued to rise over the succeeding 2 weeks to a significantly higher level at week 6. In vitro adrenal corticosterones remained unchanged throughout the experiment.

Table 12 shows the results of TLC separation of tritiated adrenal steroids after incubation of the adrenals in the presence of pregnenalone-7-^{3}H. The C/D ratio, corticosterone/pregnenalone (C/P) ratio and the deoxycorticosterone/pregnenalone (D/P) ratio did not significantly differ from controls at any time during the 6 week experiment.

Table 13 shows the organ weight-body weight ratios. No significant changes were found during the first 2 weeks of treatment although the kidney weights showed a slight increase as early as 1 week of treatment. After 3 weeks of treatment, liver, kidney and heart weight-body weight ratios were significantly increased in the treated animals. These organ weights remained significantly larger after 6 weeks and, in addition, the spleen weight-body weight ratio increased significantly at this time. As previously noted in Figure 7, there were no significant changes in total body weight during the 6 week study.

Figure 7. Food intake and average weight of male Sprague-Dawley rats fed subacutely a diet of ground Purina Chow containing 4% tech-AG. Each point represents an average ± SEM for the following numbers of animals; weeks 0 and 1, n = 20; week 2, n = 16; week 3, n = 12; weeks 4, 5 and 6, n = 8.

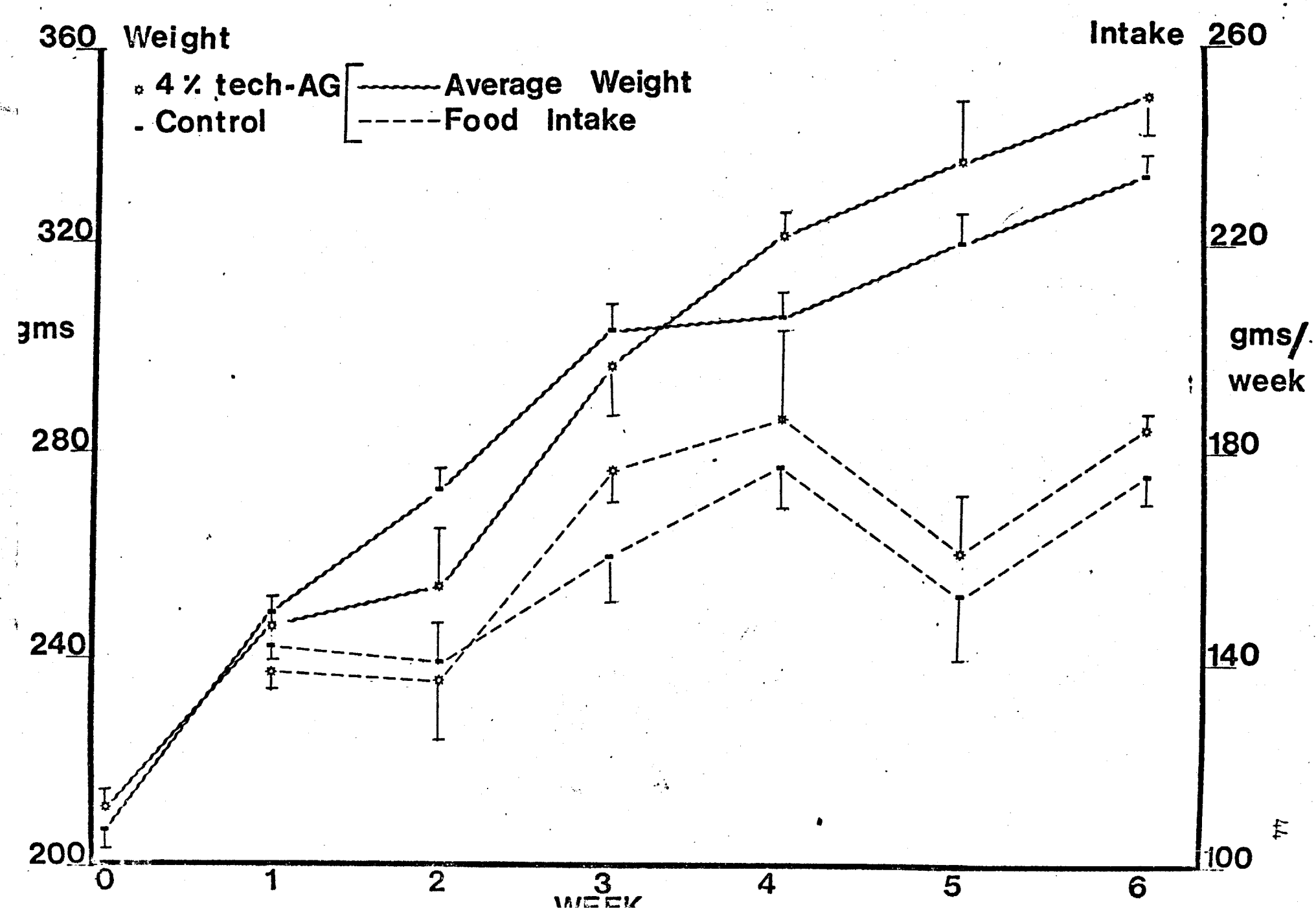

360
Weight
Intake
260
4 % tech-AG
Control
Average Weight
Food Intake
320
220
gms
gms/
week
280
180
240
140
200
100
0
1
2
3
4
5
6
WEEK

Table 11

PLASMA CORTICOSTERONE AND IN VITRO ADRENAL CORTICOSTERONE LEVELS OF MALE SPRAGUE-DAWLEY RATS FED SUBACUTELY A DIET CONTAINING 4% TECH-AG

Week	Plasma µg% ± SEM		Adrenal µg/mg ± SEM	
	Test	Control	Test	Control
Week 1 (4)[1]	33.5 ± 5.8	30.0 ± 7.4	0.21 ± 0.01	0.19 ± 0.01
Week 2 (4)	35.5 ± 5.2	30.0 ± 5.7	0.24 ± 0.01	0.26 ± 0.02
Week 3 (4)	50.8 ± 8.4	32.2 ± 6.4	0.22 ± 0.02	0.17 ± 0.01
Week 6 (8)	64.7* ± 3.8	43.6 ± 5.2	0.26 ± 0.01	0.23 ± 0.01

[1] (n) = number of samples analyzed.

* Statistically significant $p < 0.05$.

Table 12

RATIOS OF DPM RECOVERED FROM INCUBATION OF ADRENALS OF MALE SPRAGUE-DAWLEY RATS IN THE PRESENCE OF PREGNENALONE-7-^{3}H AFTER SUBACUTE FEEDING WITH 4% TECH-AG

Week	C/D ± SEM		C/P ± SEM		D/P ± SEM	
	Test	Control	Test	Control	Test	Control
Week 1 (4)[1]	2.81[2]	0.82 ± 0.08	0.040[2]	0.020 ± 0.000	0.020[2]	0.025 ± 0.000
Week 2 (4)	0.93 ± 0.18	1.43 ± 0.61	0.022 ± 0.005	0.023 ± 0.003	0.025 ± 0.005	0.021 ± 0.007
Week 3 (4)	1.20 ± 0.25	1.12 ± 0.16	0.022 ± 0.000	0.021 ± 0.000	0.021 ± 0.005	0.020 ± 0.004
Week 6 (8)	1.15 ± 0.14	1.32 ± 0.18	0.026 ± 0.000	0.030 ± 0.000	0.025 ± 0.003	0.024 ± 0.000

[1] (n) = number of animals analyzed; [2] represents value for one animal only;
C = corticosterone, D = deoxycorticosterone, P = pregnenalone.

Table 13

ORGAN WEIGHT-BODY WEIGHT RATIOS OF MALE SPRAGUE-DAWLEY RATS FED SUBACUTELY A DIET OF 4% TECH-AG

Weeks	Level	Liver[1]	Kidney[1]	Spleen[1]	Heart[1]	Testes[1]	Brain[1]	Adrenal[2]
Week 1	4% tech-AG (4)[3]	4.4 ± 0.1	0.90 ± 0.02	0.27 ± 0.01	0.36 ± 0.01	0.60 ± 0.04	1.14 ± 0.08	16.5 ± 0.6
	Control (4)	4.3 ± 0.2	0.84 ± 0.03	0.30 ± 0.01	0.34 ± 0.01	0.61 ± 0.03	1.14 ± 0.04	13.5 ± 1.4
Week 2	4% tech-AG (4)	3.6 ± 0.2	0.92 ± 0.04	0.15 ± 0.02	0.31 ± 0.01	0.63 ± 0.06	1.17 ± 0.15	19.4 ± 2.1
	Control (4)	3.7 ± 0.3	0.80 ± 0.04	0.22 ± 0.03	0.34 ± 0.03	0.52 ± 0.03	1.09 ± 0.05	16.2 ± 1.3
Week 3	4% tech-AG (4)	4.6* ± 0.2	0.97* ± 0.04	0.22 ± 0.01	0.39* ± 0.003	0.56 ± 0.04	1.21 ± 0.05	15.8 ± 1.5
	Control (4)	3.8 ± 0.1	0.77 ± 0.01	0.20 ± 0.01	0.34 ± 0.01	0.47 ± 0.03	1.01 ± 0.07	15.0 ± 1.0
Week 6	4% tech-AG (8)	4.4* ± 0.1	0.99* ± 0.03	0.22* ± 0.01	0.40* ± 0.01	0.51 ± 0.02	0.93 ± 0.03	14.7 ± 0.6
	Control (8)	3.4 ± 0.1	0.75 ± 0.02	0.19 ± 0.01	0.30 ± 0.01	0.49 ± 0.01	0.91 ± 0.03	15.0 ± 0.6

[1] gm tissue/100 gm final body weight ± SEM; [2] mg tissue/100 gm final body weight ± SEM; [3] (n) = number of animals analyzed; * statistically significant $p < 0.05$.

CHAPTER IV

DISCUSSION

This thesis describes experiments undertaken to produce hypertension in the intact rat by administration of AG and to evaluate the possibility that the hypertension produced by subacute ingestion of AG might be caused by an inhibition of the 11β-hydroxylase enzyme necessary for the conversion of DOC to corticosterone by the adrenal gland. Hypertension of this type would be similar to DOCA-induced hypertension which is enhanced by co-administration of saline (Selye et al., 1943). Levels of plasma and adrenal corticosterone and the extent of in vitro conversion of pregnenalone-7-^{3}H to DOC and corticosterone were measured in these experiments.

The failure of the first three dosing schedules to produce hypertension in the O.M. rat even after oral dosing for as long as 8 weeks and subacute feeding for 17 weeks led to the examination of two possibilities: 1) that the dosing with AG would require higher levels for longer periods, or 2) that the O. M. rat (FDA strain) was resistant to experimental hypertension of this type. The results of simultaneous dosing of O. M. and S. D. rats for 5 weeks at 1000 and 2000 mg/kg/day with tech-AG increased the probability of the second conclusion.

There have been several accounts in the literature indicating that different strains of rats have variable susceptibility to various types of experimental hypertension. In 1962, Dahl et al. successfully developed, by selective breeding, two strains of rats, one susceptible to the hypertensive effects of sodium chloride, and the other resistant. In studies designed to develop a renal hypertensive model, it was found that the S. D. rat produced the expected response in blood pressure while the Carworth Farm (C. F.) rat failed to respond (Park, 1971). With chronic DOCA administration, the C. F. rat further responded with a decrease in blood pressure. It was found that the Long Evans strain of rats also failed to respond to salt and DOCA hypertension when compared with the S. D. rat (Hall et al., 1972).

The O. M. rats used in the earlier experiments failed to show any dose response to the treatment although the blood pressure of all animals, test and control, showed a progressive rise with age until it stabilized around 140 mm Hg, a level somewhat higher than the average control level of 125 mm Hg found in the S. D. rats used in these experiments. Long term treatment (20 months) by subacute feeding of tech-AG to O. M. rats has produced erratic but small increases in blood pressure as compared to control fed rats (FDA, unpublished data). Further experimentation would be necessary to determine the true responsiveness of the O. M. rat to various forms of experimental hypertension, or even to evaluate the possibility of this strain being "resistant" to hypertension. Such studies were beyond the purpose of this thesis.

The O. M. rat dosed at 2000 mg/kg/day for 5 weeks did, however, demonstrate the expected resonse in organ weight changes, showing a significant increase in kidney and heart weights as is typical of the DOCA-saline treated animal (Hall and Ayachi, 1971) along with an increase in testes weight-body weight ratio, the significance of which is unknown.

The S. D. rat responded very rapidly to treatment with tech-AG showing a significant increase in blood pressure after 2 weeks of dosing at 1000 mg/kg/day and after 3 weeks of dosing at 2000 mg/kg/day. However, results of the corticosterone assays in the two groups differed. The animals dosed at 1000 mg/kg/day showed a significant decrease in plasma corticosterone and in the amount of corticosterone produced during <u>in vitro</u> adrenal incubation in the presence of added DOC (Incubation B). This was the response expected if, in fact, tech-AG did inhibit the 11β-hydroxylation of DOC to corticosterone. However, the animals dosed at the higher level of 2000 mg/kg/day showed a significant increase in plasma corticosterone and no change in the <u>in vitro</u> adrenal corticosterone production. Since the animals dosed at the 2000 mg/kg/day level showed early signs of toxicity, it was considered at this time that

the increased plasma corticosterone levels in these rats might have been caused by a non specific stimulation of the adrenal pituitary axis due to toxicity of AG.

Neither dose level produced any changes in the serum electrolytes and the effects on organ weight-body weight ratios were inconsistent. The animals at the lower dosage level exhibited a decrease in testes and brain weights while those at the higher level showed an increase in heart weight.

The next experiment consisted of dosing a group of male and female S. H. rats with tech-AG at the lower, less toxic, level. An increase in blood pressure in the S. H. animals appeared as early as 1 week after start of treatment in the male rats but not until after 6 weeks of treatment in the females. While neither sex showed any differences in in vitro adrenal corticosterone production as compared to controls, the females showed a significant decrease in plasma corticosterone levels. A similar trend was apparent in the male rats but this effect was not statistically significant. Furthermore, a significant increase in adrenal to body weight ratios was present in both male and female rats. These results are all consistent with the hypothesis that AG blocks the conversion of DOC to corticosterone.

The results of the in vitro incubation of the adrenals in the presence of pregnenalone-7-^{3}H failed to show the expected increase in adrenal DOC levels. In fact, the female rats showed a significant increase in the C/D ratio rather than the anticipated decrease due to a high DOC level. While this result seemed to indicate a significant increase in adrenal corticosterone, this was inconsistent with the actual measurement of adrenal corticosterone, by the spectrofluorescent method, which showed no change in the corticosterone of these animals as compared to controls. Examination of both the C/P and D/P ratios also indicated an increase in the adrenal corticosterone level. The other explanation for this ratio could be an increase in the metabolism

of the pregnenalone-7-^{3}H to some other metabolic product along with a decrease in DOC produced in this system. Examination of the actual DPM offered no further solutions.

The male rats had a significant increase in serum sodium. The S. H. rat, test and control alike, showed a significantly lower serum potassium level than found in either O. M. or S. D. rats. Baer et al., in 1972, reported that S. H. rats had a mean depression of plasma potassium concentration of approximately 10% as compared to normal Wistar rats. The S. H. rats used in this study showed a mean depression of serum potassium concentration of approximately 55% as compared to the other two unrelated strains used.

In addition to the effects of treatment with AG on the adrenal weight-body weight ratios, both the male and female S. H. rat showed significant increases in kidney and heart weights. The females, however, also showed increases in liver, brain and spleen weights. These changes in the female again indicated the possibility of toxic effects due to treatment with tech-AG.

The failure to obtain the anticipated increase in adrenal DOC along with the decreased corticosterone at the low dose levels and the increased corticosterone at the higher level led to the examination of the possibility of transient changes in both of these adrenal steroids. In 1972, Brown et al. reported that animals subjected to adrenal regeneration hypertension displayed levels of plasma DOC ranging from near unmeasurable levels immediately after adrenal enucleation to six times that of controls at 3 weeks post-operatively with a gradual decline to normal at 7 weeks. Other workers found similar changes. Plasma corticosterone levels in animals with this type of hypertension ranged from subnormal (Brogi and Pelligrino, 1959) to normal at 16 to 20 days post-operatively (Fortier and DeGroot, 1959; Ichii et al., 1968).

In order to examine this possibility, a group of male S. D. rats was fed a diet of 4% tech-AG for a 6 week period. Randomly selected animals in

this experiment were sacrificed after 1, 2, 3 and 6 weeks of treatment and plasma and adrenal corticosterone levels and adrenal DOC production in the presence of pregnenalone-7-^{3}H were examined. While a significant increase in blood pressure was evident after 1 week of treatment, a decrease in plasma and adrenal corticosterone, as previously seen in the S. D. rat dosed at 1000 mg/kg/day, never developed. Instead, these animals showed a progressive increase in plasma corticosterone to a significantly high level after 6 weeks of treatment. Likewise, the adrenal C/D ratio remained unchanged over the 6 weeks of treatment. Consistent with the increased blood pressure, saline polydipsia was evident after 3 weeks of treatment along with a significant increase in kidney and heart weights. After 6 weeks of treatment, a significant increase in liver and spleen weights was also evident. While these animals were consuming a dose of AG equivalent to 3200 mg/kg/day initially to 3000 mg/kg/day by 6 weeks, a dose higher than that previously shown to produce toxicity by oral intubation, no signs of toxicity were visible at this dose when it was fed in the diet.

The results of these studies appear to rule out the hypothesis that AG produces hypertension in the intact rat by inhibition of the 11β-hydroxylation of DOC to corticosterone. That AG does produce hypertension otherwise resembling that which is DOCA-saline induced was shown. A number of reports have shown that another adrenal corticosteroid may be involved in various types of experimental hypertension. In 1961, Peron isolated a steroid from rat adrenals which he identified as 18-hydroxydeoxycorticosterone (18-OH-DOC). Birmingham et.al. in 1968 were able to produce the same degree of sodium retention and antidiuresis in rats with equal doses of DOC and 18-OH-DOC. Rapp and Dahl, in 1971, showed that their rats specifically bred for sensitivity to the hypertensive effects of sodium chloride produced twice as much 18-OH-DOC by in vitro adrenal incubation in the presence of tritiated DOC than those rats

showing resistance to salt hypertension. This higher 18-hydroxylation was exactly offset by a correspondingly lower 11β-hydroxylation. The peripheral plasma 18-OH-DOC concentration in the susceptible strain was approximately twice that in the resistant strain while plasma corticosterone levels did not differ. Likewise, various reports have indicated that in vitro adrenal 18-OH-DOC production (Birmingham et al., 1968) and levels of 18-OH-DOC in peripheral plasma (Shapiro and Peron, 1973) and adrenal venous effluent (Melby et al., 1972) were significantly increased in the later stages of adrenal regeneration hypertension, although, the findings with respect to the peripheral plasma 18-OH-DOC levels were disputed by Rapp (1970). That this steroid might play a role in the hypertension caused by AG cannot be ruled out.

Finally, the steroid-like structure of AG leaves open the possibility that the compound itself might have a direct DOCA-like effect on the kidney, resulting in hypertension, electrolyte imbalances and increased kidney and heart weights along with decreased levels of aldosterone excretion and plasma renin activity that have characterized this type of hypertension.

SUMMARY

Ingestion of AG, a major component of licorice extract and of licorice itself, over long periods of time has been shown to produce a clinical syndrome known as pseudoaldosteronism and characterized by a progressive increase in arterial blood pressure, hypokalemia, hypernatremia and edema with decreased levels of aldosterone excretion and decreased plasma renin activity.

This thesis has described experiments designed to produce hypertension in the intact rat by subacute dosing with tech-AG and to determine if this hypertension might be due to an inhibition of the 11β-hydroxylation of DOC to corticosterone by the adrenal gland.

Daily oral dosing and subacute feeding of pure- and tech-AG, respectively, to the O. M. rat (FDA strain) failed to produce any change in blood pressure. Simultaneous dosing of O. M. and S. D. rats at 1000 and 2000 mg/kg/day with tech-AG produced a significant increase in blood pressure in the S. D. rat with little to no response in the O. M. rat. This led to the consideration of the possibility that the O. M. rat might be resistant to hypertension, at least to that produced by AG.

Dosing with tech-AG at 1000 mg/kg/day in both S. D. and S. H. rats has produced hypertension, decreased plasma corticosterone and increased kidney and heart weights. However, feeding a 4% diet of tech-AG or dosing at 2000 mg/kg/day in the S. D. rat has produced increased plasma corticosterone levels along with the hypertension and the increased kidney and heart weights.

Neither daily dosed S. H. rats nor subacutely fed S. D. rats showed any evidence of increases in adrenal DOC production as would be expected if AG acted by inhibiting 11β-hydroxylase.

Whether the hypertension produced by long term intake of tech-AG is due to another steroid, such as 18-OH-DOC, or to a direct effect of the compound on the kidney remains to be assessed.

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